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2017-07-04

Yang , Z , Piironen , V I & Lampi , A-M 2017 , ' Lipid-modifying enzymes in oat and faba bean ' , Food Research International , vol. 100 , pp. 335-343 . https://doi.org/10.1016/j.foodres.2017.07.005

http://hdl.handle.net/10138/310954
https://doi.org/10.1016/j.foodres.2017.07.005

Lipid-modifying enzymes in oat and faba bean

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Abstract

The aim was to study lipase, lipoxygenase (LOX) and peroxygenase (POX) activities in oat and faba bean samples to be able to evaluate their potential in formation of lipid-derived off-flavours. Lipase and LOX activities were measured by spectroscopy, and POX activities via the formation of epoxides. An ultra-high performance liquid chromatography method was developed to study the formation of fatty acid epoxides. The epoxides of esters were measured by gas chromatography. Mass spectroscopy was used to verify the identity of the epoxides. Both oat and faba bean possessed high lipase activities. In faba bean, LOX catalysed the formation of hydroperoxides, whose break-down products are the likely cause of off-flavours. Since oat had low LOX activity, autoxidation is needed to initiate lipid oxidation. Oat had high POX activity, which is able to convert hydroperoxides to epoxy and hydroxy fatty acids that could contribute significantly to off-flavours. POX activity in the faba bean was low. Thus, in faba bean volatile lipid oxidation products could rapidly be formed by LOX, whereas in oat reactions are slower due to the need of autoxidation prior to further reactions.

Keywords: Oat; Faba bean; Lipase; Lipoxygenase; Peroxygenase; Off-flavour; Analysis of fatty acid epoxides

1. Introduction

Oat (*Avena sativa*) and faba bean (*Vicia faba L.*) are good candidates when new sources of plant proteins and contributors to the sustainable development in agricultural and food systems are looked for. Oat has long been recognised as a valuable source of many nutrients for humans and animals (Decker, Rose, & Stewart, 2014). The faba bean is one of the leading grain legumes in the world, and it is mainly used as a rich source of protein, but it also contains lots of starch (Crépon et al., 2010; Lizarazo et al., 2015). One problem associated with using plant proteins in foods is formation of lipid-derived off-flavours, which diminish the consumer acceptance of these products. Some of the off-flavours develop immediately through the action of enzymes, whereas some occur only after long-term storage. To control enzymatic reactions, the inactivation of lipid-modifying enzymes by heat treatment is essential (Decker et al., 2014; Jiang et al., 2016; Lehtinen, Kiiliäinen, Lehtomäki, & Laakso, 2003; Roland, Pouvreau, Curran, van de Velde, & de Kok, 2017). However, overly severe treatments should be avoided because they may cause problems in the sensory and technological properties of the products.

One lipid-modifying enzyme is lipase, which liberates free fatty acids (FFAs) from their esters. The liberated fatty acids may be oxidised chemically or enzymatically into odourless and tasteless hydroperoxides. The endogenous lipases will start hydrolysing acyl lipids as soon as the seed structure breaks down and the enzyme comes into contact with its substrates. The function of lipases during the processing and storage of oat has been well characterised (Decker et al., 2014; Lehtinen et al., 2003). A lipase has also been isolated from small faba bean (Dundas, Henderson, & Eskin, 1978) and a sequential hydrolytic pathway has been proposed (Henderson, Shambrock, & Eskin, 1981). However, little is known of lipase activity and its impact in faba bean.

Lipoxygenase (LOX) is the enzyme responsible for the formation of hydroperoxides, which in turn can further react to form volatile off-flavours, such as hexanal, or non-volatile products, such as oxoacids and dimers (Gardner, 2003) through several chemical and enzymatic pathways. LOX activity in legumes is regarded as responsible for the undesirable "beany flavour" through the break-down of its hydroperoxide products (Gardner, 2003; Roland et al., 2017). The faba bean has been categorised as possessing medium-level LOX activity among legumes (Chang & McCurdy, 1985). Two LOX isoenzymes have been characterised from the faba bean (Clemente, Olías, & Olías, 2000). Much less is known about LOX activities in cereal grains. In a review paper, Lehtinen and Kaukovirta-Norja (2011) reported that LOX activity in oat was lower than in, for example, wheat and barley.

Peroxygenase (POX) catalyses the hydroperoxide-dependent conversion of unsaturated fatty acids into non-volatile flavour compounds, namely epoxy and hydroxy fatty acids (Hamberg & Hamberg, 1996). The POX pathway begins when one of the oxygens of a fatty acid hydroperoxide is transferred to an unsaturated fatty acid, yielding a hydroxy fatty acid and an epoxy fatty acid. These compounds have been suggested to be responsible for the formation of the bitter taste in aged oat products (Hamberg & Hamberg, 1996; Doehlert, Angelikousis, & Vick, 2010). POX has been isolated from oat seeds (Hamberg & Hamberg, 1996). Currently, oat POX has important potential in the production of fatty acid epoxides in the chemical industry (Piazza, Nuñez, & Foglia, 2003). Hamberg and Fahlstadius (1992) have also observed hydroperoxide-dependent epoxidation of unsaturated fatty acids in faba bean. However, little research has been done on the POX activity in either oat or faba bean foods.

Although detrimental effects of lipase on oat lipids and of LOX on faba bean lipids are well described (Piazza, Bilyk, Brower, & Haas, 1992; Clemente et al., 2000; Lehtinen & Kaukovirta-

77 Norja, 2011), there is very little knowledge on the overall effects of the lipid-modifying enzymes on
78 oat and faba bean products, and the levels and variation of enzyme activities present in the seeds.
79 Understanding and controlling the lipid-modifying enzymes is essential to prolong the shelf life of
80 the products and raise consumer acceptance towards them.
81
82 The aim of the study was to understand the potential of lipase, LOX and POX activities present in
83 oat and faba bean seeds and to evaluate their possible contribution to the formation of compounds
84 related to lipid-derived off-flavours. The enzyme activities were studied in seeds from several
85 cultivars and growing seasons. Lipase and LOX activities were measured using spectrophotometric
86 methods, whereas methods to study POX activities in various substrates were developed.
87
88 **2. Materials and methods**
89 *2.1. Chemicals and standards*
90 Substrates for the enzyme activity analyses, including methyl oleate (purity > 99%), methyl
91 linoleate (purity > 99%), methyl linolenate (purity > 99%), triolein (purity > 99%), oleic acid
92 (purity > 99%), linoleic acid (purity > 99%), linolenic acid (purity > 99%), as well as the internal
93 standards, nonadecanoic acid (purity > 99%) and its methyl ester (purity > 99%), were purchased
94 from Nu-Check Prep, Elysian, MN, USA. Cumene hydroperoxide (80%) and para-nitrophenyl
95 butyrate (purity ≥ 98%) were obtained from Sigma-Aldrich (St. Louis, USA). The epoxy stearic
96 acid used for the quantification of epoxy fatty acids was obtained from Santa Cruz Biotechnology®
97 (Texas, USA). All other reagents were purchased from Sigma-Aldrich and Merck (Darmstadt,
98 Germany). Water was purified using the Milli-Q system (Millipore Corp., Bedford, MA, USA).
99
100 *2.2. Oat and faba bean samples*

Commercial oat flour from the Raisio Group (Nokia, Finland) and faba bean flour milled from the cultivar "Kontu", grown in the year 2011 at the Viikki Experimental Farm (Lizarazo et al., 2015), were used as in-house reference samples to verify the analytical level of the measurements. The oat flour was used for POX activity measurements, and the faba bean flour for lipase and LOX activity measurements. Oat grain samples (cultivars Akseli, Alku, Meeri and Steinar) were provided by Boreal Plant Breeding Ltd. (Jokioinen, Finland). They were grown in the cultivation years 2012, 2013 and 2014 in the same area in southern Finland and were stored at 10–15 °C. The faba bean samples (cultivars Kontu, Alexia, Fatima and SSNS-1) were obtained from the Viikki Experimental Farm of the University of Helsinki in southern Finland from three cultivation years. Samples from the years 2011 and 2015 were stored at 5 °C, while the samples from the year 2010 were stored at room temperature (RT). Detailed information on the faba bean samples and weather conditions was given by Lizarazo et al. (2015). All seed samples were milled using a Centrifugal Mill ZM200 (Retsch, Haan, Germany) fitted with a 0.5-mm sieve, after which the samples were immediately transferred to a freezer and stored at −20 °C before analysis.

2.3. Enzyme extraction from oat and faba bean flour

Enzymes were extracted from the milled oat and faba bean samples. The flours (2.5 g) were mixed with 23 ml of cold 0.1 M potassium phosphate buffer (pH 6.7) in centrifugation tubes, vortexed (4 x 10 s) and kept on ice for 1 h. The slurries were centrifuged at 4 °C for 10 min at 9000 x g (Sorvall RC5C, SLA-1500 rotor), the sediments were discarded and the supernatants were centrifuged for another 10 min. The supernatants were kept on ice before being used for enzyme activity analyses. Each sample was extracted in duplicate and one in-house reference sample was included in each extraction batch. All enzyme activity measurements were done in triplicate from each extract.

2.4. Lipase activity measurement

Lipase activity was measured using a spectrophotometric method with para-nitrophenyl butyrate as a substrate (Brunschwiler, Heine, Kappeler, Conde-Petit, & Nyström, 2013). The reaction was initiated by adding sample extracts into fresh substrate solutions of 2 mM para-nitrophenyl butyrate in 50 mM potassium phosphate buffer containing 0.1% Triton X-100 (pH 8.0). To achieve a minimal slope (dA/min of 0.03), 20 µl of faba bean extract and 80 µl of oat extract were mixed with the substrate solution (total volume of 1.00 ml). The increase in the absorption at 405 nm during 150 s was measured with the ultraviolet spectrometer (Lambda 25 UV/Vis, Perkin Elmer Inc., USA). The molar extinction coefficient value of 16.05 mM^{-1} cm^{-1} for hydrolysed para-nitrophenol was used to calculate the results. Lipase activity was given as µmol min^{-1} g^{-1} flour.

2.5. LOX activity measurement

LOX activity was measured by a spectrophotometric method using linoleic acid as the substrate (Jiang et al., 2016). For the LOX assays, 200 µl of the substrate solution with 10 mM of linoleic acid, 200 µl of the sample extracts and 2.6 ml of 0.1 M potassium phosphate buffer (pH 6) were mixed and incubated for 3 min at 30 °C in a water bath. The reaction was stopped by adding 3 ml of 0.1 N KOH solution and the absorbances were measured at 234 nm (Lambda 25 UV/Vis, Perkin Elmer Inc., USA). The results were calculated using the molar absorptivity of conjugated dienes (ε = 26 000 l/ mol cm). The LOX activity was expressed as mmol min^{-1} g^{-1} flour.

2.6. Method development for POX activity measurement

The POX activity was studied by measuring epoxide formation. FFAs, their methyl esters and triolein were used as substrates to observe the specificities of the enzyme in the oat and faba bean samples. Two methods were developed, because epoxides of esters could be analyzed by GC whereas it was not applicable to epoxides of free fatty acids, for which an UHPLC method was developed.

151

152 *2.6.1. GC-FID/MS method for studying fatty acid methyl esters and triolein as substrates*

153 Methyl oleate, methyl linoleate and methyl linolenate were used as methyl ester substrates for POX.
154 Aliquots of 435 µl of mixtures containing one of the substrates (ca. 3 mg/ml) and the internal
155 standard, methyl nonadecanoate (1.4 mg/ml) in heptane, were first evaporated under N_2 at 35 °C,
156 and then the residues were dissolved in 350 µl of 1 % Tween 20 in Milli-Q water by vortexing. To
157 the substrate solutions 1.75 ml of 100 mM HEPES at pH 7.0, 900 µl of Milli-Q water and 500 µl of
158 the sample extracts were added. To start the enzymatic reaction, 7 µl of 8 % cumene hydroperoxide
159 diluted in ethanol was added and the tubes were mixed by inverting with a rotator for 1 h at RT,
160 after which the reaction was stopped by adding 5 ml of methanol. The lipids were extracted twice
161 with 10 ml of diethyl ether and the extracts were washed with 5 ml of saturated NaCl in Milli-Q
162 water. Aliquots of 10 ml were dried under N_2 evaporation at 35 °C. The residues were immediately
163 re-dissolved into 1 ml of heptane. To study triolein as the substrate, the assay was carried out
164 following the same steps as mentioned above, except that the final dried residues were re-dissolved
165 into 1.5 ml of heptane, after which the substrates and the products were methylated by alkaline
166 transesterification (Christie, 1993).

167

168 Finally, fatty acid methyl esters and their epoxides were analysed by GC. A GC-FID (Agilent
169 6890N, USA) equipped with a fused silica capillary column Omegawax™ l250 (30 m × 0.25 mm ×
170 0.25 µm, SUPELCO®, Bellefonte, USA) was used for quantitative analysis, and a GC (HP 6890
171 series, Agilent Technologies Inc., Wilmington, DE, USA) with an MS detector (Agilent 5973
172 Network, Agilent Technologies Inc., Wilmington, DE, USA) equipped with the same capillary
173 column was used for the identification of the epoxides. In the GC-FID analysis, helium was used as
174 a carrier gas at a flow rate of 1.1 ml/min and 1-µl samples were injected in a split mode (1:15) at
175 240 °C. The oven temperature was programmed to increase from 160 °C (with a 1-min initial hold)

with a 4 °C/min to 240 °C (with a 15-min final hold). The GC-MS analysis was conducted similarly, except that the split ratio was 1:20, and the oven temperature was programmed to increase from 150 °C (with a 2-min initial hold) with a 4 °C/min to 240 °C (with a 20-min final hold). The ionisation energy of MS was 70 eV, the ion source temperature was 230 °C and the mass range of *m/z* 40–450 was scanned. The epoxides and substrates were quantified using an internal standard method and assuming equal FID responses for all compounds. Finally, the results were expressed as a % of the epoxide(s) formed and as a % of the substrate residues left from each substrate.

2.6.2. UHPLC-ELSD and UHPLC-Q-TOF methods for studying free fatty acids as substrates

FFAs at three unsaturation levels – oleic acid, linoleic acid and linolenic acids – were used as substrates (ca. 3 mg/ml) for POX. Nonadecanoic acid used as an internal standard (3.16 mg/ml) was included in the substrate mixtures. The mixtures were incubated, and thereafter the lipids were extracted with diethyl ether, as described in Section *2.6.1*. After evaporation, the residues were immediately re-dissolved into 1 ml of isopropanol and 1 ml of methanol. Finally, the fatty acids and their epoxides were analysed by UHPLC with a method developed in this study.

For the quantification of the lipids, a UHPLC-ELSD instrument was used, and for the identification of the epoxides, a UHPLC-Q-TOF instrument was used. The UHPLC-ELSD instrument consisted of a UHPLC (Waters Acquity, Milford MA, USA) coupled with an ELSD detector (Waters Acquity, Milford, MA, USA). The FFAs and epoxides were separated with a reversed-phase Acquity UPLC® HSS T3 column (1.8 µm, 2.1 × 150 mm, Waters, Ireland) using gradient elution consisting of a mixture of Milli-Q H_2O and 0.05% acetic acid (solvent A) and of methanol with 0.05% acetic acid (solvent B) at a flow rate of 0.3 ml/min at 30 °C. The 15-min elution program with the two solvents (solvent A:solvent B, vol%:vol%) was as follows: 0–0.5 min (25:75); 0.5–9 min (25:75 to 2:98); 9–12 min (2:98); 12–12.5 min (2:98 to 25:75); and 12.5–15 min (25:75). The

ELSD drift tube temperature was set to 50 °C and the gain to 500. Nebulisation was performed with filtered air at 40.0 psi, and the cone and desolvation gas flows were 100 and 1000 l/Hr, respectively. The epoxide contents were calculated based on the standard curves of the second-order equations made from the 9,10-epoxystearic acid standard and internal standard nonadecanoic acid. The results were expressed as a % of the epoxide(s) formed and as a % of the substrate residues left from each substrate.

The identification of the fatty acid epoxides was carried out in a negative ion mode on a UHPLC-Q-TOF (Acquity I class, Milford, MA, USA) with an electrospray ionisation (ESI) interface, coupled with a SYNAPT G2-Si Mass Spectrometer. The column, eluents and elution program were the same as in the UHPLC-ELSD instrument. The scanning was carried out in the mass range of *m/z* 50–1200. The instrumental settings were as follows: capillary voltage 2.5 kV, sampling cone voltage 40 V, source offset 80V, source temperature 100 °C, desolvation temperature 500 °C, desolvation gas flow 1000 l/h, nebuliser gas flow 6.2 bar, cone gas flow 100 l/h, trap collision energy 4 eV, ramp for MS/MS trap collision energy 10–70 eV, trap gas flow 2 ml/min and a scan time of 0.3 s.

2.7. Statistical analysis

All results were expressed as mean values ± standard deviations of six replicate samples. Statistical analysis was performed using SPSS version 22 (IBM SPSS Statistics, USA). A value of $p < 0.05$ was considered statistically significant. To study the effects of cultivars and sample years and their interactions on the enzyme activities, the results were subjected to a two-way analysis of variance (ANOVA), and thereafter to Tukey tests to identify homogenous groups of samples. Figures were drawn using OriginPro 8.6 (OriginLab Corporation, USA).

3. Results and discussion

3.1. Lipase and lipoxygenase activities in oat and faba bean

Both the oat and faba bean samples had significant lipase activities when measured using para-nitrophenyl butyrate as the substrate. In oat, the activities ranged between 0.41 ± 0.02 and 0.77 ± 0.03 µmol min^{-1} g^{-1} flour (Fig. 1a), while in faba bean, they ranged between 4.44 ± 0.34 and 7.51 ± 0.44 µmol min^{-1} g^{-1} flour (Fig. 1b). In both species, the activities varied almost two-fold among cultivars and samples from different years. The activity in the faba bean samples was ten-fold that of the oat samples. Lipase activity and its impact on oat have been well characterised (Decker et al., 2014; Lehtinen et al., 2003; Piazza et al., 1992), while only the presence and the basic characteristics of the enzyme in faba bean have been identified (Dundas et al., 1978; Henderson et al., 1981). The faba bean in-house reference sample gave a stable activity value of 6.72 ± 0.12 µmol min^{-1} g^{-1} flour (n = 13), showing that the level of analysis was stable and that the results could be compared throughout the study.

In the oat samples, there were differences in lipase activity among the cultivars (ANOVA, $F_{3.60}$ = 125.8, $p < 0.05$) and the cultivars were divided into three homogenous groups (Tukey HSD, $p < 0.05$). The lowest lipase values were found in cultivar Alku, with an average of 0.54 ± 0.11 µmol min^{-1} g^{-1} flour, while the highest occurred in cultivar Akseli, with an average of 0.71 ± 0.08 µmol min^{-1} g^{-1} flour. Ekstrand et al. (1992) found a comparable variation in lipase activity among three cultivars, which ranged 220–268 U/g, but also greater variations have been found (Miller, Fulcher, & Altosaar, 1989; Hu, Wei, Ren, & Zhao, 2009). The lipase activities from the oat samples from 2012–2014 were also different (Anova, $F_{2.60}$ = 553.6, $p < 0.05$) and samples from each year differed from the others (Tukey HSD, $p < 0.05$). Lipase activities increased from 2012 to 2014, resulting in average values of 0.48 ± 0.08, 0.64 ± 0.10 and 0.72 ± 0.05 µmol min^{-1} g^{-1} flour, respectively. It may indicate that long-term storage could have negatively influenced the activity. In addition, the growing and harvesting conditions in different years might have had an effect. However, the trend

among the sample years was not always similar and there was a statistically significant interaction between the cultivars and sample years (Anova, $F_{6.60} = 28.7$, $p < 0.05$). For instance, the activity in cultivar Akseli was higher in samples from year 2013 than from year 2014. In summary, the cultivar and the sample year were observed to have an effect on the oat lipase activity.

For faba bean lipase activities, there were significant differences among the cultivars (Anova, $F_{3.60} = 259.1$, $p < 0.05$) (Fig. 1b) and all the cultivars differed from each other (Tukey HSD, $p < 0.05$). The highest activities were found in cultivar Kontu, with an average of 7.18 ± 0.45 μmol min^{-1} g^{-1} flour, and the lowest in cultivar Alexia, with an average of 4.54 ± 0.28 μmol min^{-1} g^{-1} flour. The lipase activities for cultivars Fatima and SSNS-1 were 6.85 ± 0.23 and 6.22 ± 0.29 μmol min^{-1} g^{-1} flour, respectively. There was more variation in lipase activities among the faba bean cultivars than among the oat cultivars. The activities in the faba bean samples were not statistically significantly different among different cultivation years (Anova, $F_{2.60} = 2.4$, $p > 0.05$), unlike they varied in oat. The average lipase activity values for the faba bean samples were 6.16 ± 1.10, 6.28 ± 1.11 and 6.09 ± 0.94 μmol min^{-1} g^{-1} flour for cultivation years 2010, 2011 and 2015, respectively. Large variations within the years further support that the main effect in the variation among the faba bean samples was from the cultivar. The very high lipase activities and the high variation among the faba bean cultivars found in this study are to be taken into account when processing faba bean, because without inactivating the lipases, TAGs and other acyl lipids are prone to lipolysis as soon as the intact seed structures are broken. It should be recognised that the actual lipase activities towards faba bean lipids might be lower, as the faba bean lipase was earlier shown to be more active towards esters of short-chain fatty acids than of vegetable oils (Dundas et al., 1978), and in this study, the substrate was a butyric ester. Oat lipase, on the other hand, has substrate specificity to long chain fatty acids such as oleic, linoleic and linolenic acids (Piazza et al., 1992).

LOX activities were observed only in the faba bean samples (Fig. 1c), whereas in the oat samples, the activity could not be measured. The presence of LOX in faba bean, as in other legumes, is generally acknowledged (Gökmen, Bahceci, & Acar, 2002; Hildebrand & Kito, 1984), and minor LOX activities have been found in oat seeds (Lehtinen & Kaukovirta-Norja, 2011). LOX activities among the faba bean samples ranged from 0.219 ± 0.009 to 0.330 ± 0.004 mmol min^{-1} g^{-1} flour (Alexia 2011 and 2010). The levels of LOX activity found in this study were comparable to the level (0.44 mmol min^{-1} g^{-1} flour) that was found previously in Finland (Jiang et al., 2016). The faba bean in-house reference sample, gave a LOX activity value of 0.33 ± 0.01 mmol min^{-1} g^{-1} flour (n = 9). Therefore, the analysis level during this study was stable and the results could be compared.

There were significant differences in the LOX activities of faba bean cultivars (Anova, $F_{3.60} = 50.2$, $p < 0.05$) and three homogenous groups among the cultivars could be determined (Tukey HSD, $p < 0.05$). LOX values for cultivars Kontu and Fatima were the highest, with average values of 0.309 ± 0.027 and 0.295 ± 0.008 mmol min^{-1} g^{-1} flour, respectively, while the lowest average value was for cultivar Alexia at 0.274 ± 0.058 mmol min^{-1} g^{-1} flour. Although statistically significant differences among the cultivars were observed, the value for Alexia was only 11% lower than those of Kontu and Fatima. Comparably, the variation in LOX activities among three faba bean cultivars was only < 10% (Chang & McCurdy, 1985). The sample year had a significant effect on the LOX activity of the faba bean samples (Anova, $F_{2.60} = 34.2$, $p < 0.05$) and the samples from the year 2011 produced lower values of 0.278 ± 0.040 mmol min^{-1} g^{-1} flour than those from the years 2010 and 2015 at 0.299 ± 0.032 and 0.300 ± 0.029 mmol min^{-1} g^{-1} flour, respectively (Tukey HSD, $p < 0.05$). Yet the average difference among the years was only 7%. However, the interaction between the cultivar and the year was statistically significant (Anova, $F_{6.60} = 72.8$, $p < 0.05$), with cultivar Alexia having the greatest variation and the cultivars Kontu and Fatima being the most stable ones. The highest LOX activity in Alexia was 50% greater than the lowest activity that was found, whereas in Kontu and

Fatima, the differences were only 15%. In conclusion, there is LOX activity in faba bean, and the activity is affected both by the cultivar and the sample year as well as their interactions.

3.2. Analysis of epoxides from fatty acid methyl esters

All the fatty acid methyl esters studied could be converted to epoxides by oat POX, and these epoxides were used to identify epoxides isolated from esterified fatty acids. Methyl oleate, linoleate and linolenate and their epoxides were well separated by the GC-FID method (Fig. 2, combined from the chromatograms of the three substrates).To identify the epoxides, relative retention times (R_r) and selected fragments were obtained by the GC-MS method (Table 1). The R_r values of the epoxides were 1.332–1.423. Thus, the later eluted epoxides were clearly separated from the earlier eluted fatty acids with R_r values of 0.913–1.038.

The epoxides of the fatty acid methyl esters decomposed during ionisation to numerous fragments and the molecular ions (M^+) could hardly be detected. The only epoxide that was built up from methyl oleate was identified as methyl 9,10-epoxyoctadecanoate with two major fragments built up after α-scissions to the epoxide group (Table 1; Fig. 2), as also presented by Christie (2014). Most of the fragments of epoxides from methyl linoleate and linolenate were built up after cleavage of the methanol group and α-scission to the epoxide group followed by rearrangements. Two epoxides were formed from methyl linoleate (Fig. 2). The first eluting epoxide was identified as methyl 12,13-epoxy-octadec-9-enoate and the second as methyl 9,10-epoxy-octadec-12-enoate (Table 1; Fig 2). Both of them produced the fragment of *m/z* 279 characteristic for the cleavage of the methanol group, and more specific fragments of, e.g. *m/z* 207 and 168 which indicated the position of the epoxide group (Table 1). The mass spectra obtained had similar fingerprints that have been found earlier (Christie, 2014; Meesapyodsuk & Qiu, 2011). Epoxides produced from methyl linolenate could not be fully separated from each other by GC and the mixture of epoxides resulted

in a wide GC peak producing lots of fragment ions (Table 1; Fig. 2). No di-epoxy fatty acid methyl esters were observed. Using the R_r values from the GC/MS analyses, the epoxides from different fatty acid methyl esters could be identified via GC/FID analysis and used for the quantification of epoxide formation in POX assays.

3.3. Analysis of epoxides from free fatty acids

All the FFAs and their epoxidation products could be separated from each other and from the internal standard nonadecanoic acid within 15 min using a reversed-phase UHPLC method, except for the isomeric products from linolenic acid that co-eluted (Fig. 3). With the UHPLC-Q-TOF instrument, the R_r values of the epoxides were 0.532–0.649 and for the FFAs 0.732–0.869 (Table 1). In both cases, the more unsaturated an epoxide or a FFA was, the earlier it eluted. The epoxidation products were identified by UHPLC-Q-TOF with ESI in the negative mode, which produced deprotonated molecular ions $[M - H]^-$ as precursor ions. Characteristic fragments of $m/z >$ 150 were selected to structural identification of regio-isomers (Table 1). Each epoxide produced a fragment that was 18 mass units less than its precursor ion $[M - H - H_2O]^-$, verifying a loss of water from all the epoxide precursor ions during fragmentation. Only mono-epoxy fatty acids were formed during incubation.

One epoxide was found from oleic acid, and it was identified as 9,10-epoxystearic acid based on precursor ions and fragments formed after scission of the carbon-carbon bond of the epoxide group (Table 1; Fig. 3). Similar mass spectra were obtained from the standard used in this study and also in an earlier study using HPLC-ESI-MS (Orellana-Coca, Adlercreutz, Andersson, Mattiasson, & Hatti-Kaul, 2005). Two epoxides were found to be built up from linoleic acid and were identified as 12,13-mono-epoxy-9-octadecenoic acid and 9,10-mono-epoxy-12-octadecenoic acid (Table 1, Fig. 3). The position of the epoxy group was deduced based on the scission of the carbon-carbon of

351 the epoxide and the position of the double bond on the cleavage of the carbon-carbon bond on the
352 side of the double bond (Orellana-Coca et al., 2005; Murphy, 2015).
353
354 Only one linolenic acid epoxide peak with three poorly separated isomers was present when using
355 UHPLC-Q-TOF. There was one single precursor ion from the mono-epoxide octadecadienoic acid
356 isomers, while it decomposed into several negative fragment ions (Table 1, Fig. 3). After studying
357 the fragments as was done with epoxides from linoleic acid, the first one was identified as 9,10-
358 mono-epoxy-12,15-octadecadienoic acid epoxide and the second as 15,16-mono-epoxy-9,12-
359 octadidecenoic acid. The third was tentatively identified as 12,13-mono-epoxy-9,15-octadidecenoic
360 acid. The elution order of the three epoxides presented here is in line with the elution order
361 presented by Orellana-Coca et al. (2005).
362
363 In summary, as the newly developed UHPLC method could reliably separate the epoxides from the
364 fatty acids (Fig. 3), the UHPLC-ELSD method could be used to quantify the formation of the
365 epoxides of fatty acids and used to study POX activity in the extracts.
366
367 *3.4. Peroxygenase activity and substrate specificities in oat and faba bean*
368 POX activity measurements in oat and faba bean using cumene hydroperoxide as the oxygen donor
369 on different lipid substrates and analysed via the formation of epoxides showed great differences
370 between the species. The oat samples had high activity whereas no activity was found in the faba
371 bean samples. In earlier studies, POX or epoxygenase activity has been found in both oat (e.g.
372 Hamberg & Hamberg, 1996) and faba bean preparates (Hamberg & Fahlstadius, 1992), but its
373 products have only been studied in oat seeds and flours (Doehlert et al., 2010). The oat in-house
374 reference sample gave repeatable results using methyl oleate when studying fatty acid esters as
375 substrates, and using oleic acid when studying FFAs as substrates. The methyl-9,10-

376 epoxyoctadecanoate production was 14.2% ± 1.2% (n = 18) and 9,10-epoxyoctadecanoic acid
377 production was 54.9% ± 3.0% (n = 18), which means that the analysis levels using both classes of
378 substrates and analytical methods were stable and the results within the classes could be compared.
379 To our knowledge, this study is the first one to compare POX activities in different oat cultivars and
380 sample years, as well as to use formation of epoxides as the indicator of POX activity.
381
382 The POX in the oat samples could use fatty acid methyl esters and FFAs as substrates whereas no
383 epoxides were built up from triolein (Tables 2 and 3), which means that the TAGs were not suitable
384 substrates for oat POX. In line with our results, FFAs and methyl esters have been reported as the
385 more preferred substrates for oat POX than phospholipids (Meesapyodsuk et al., 2011). However,
386 as oat contains lipase activity, unsaturated fatty acids from acylglycerols may be subjected to
387 peroxygenation after being hydrolysed. The proportion of epoxides measured after incubation
388 varied from 19.1%–42.0% in methyl esters and from 34.6%–70.7% in fatty acids, indicating that oat
389 POX preferred FFAs as substrates over fatty acid methyl esters. When comparing the level of
390 unsaturation of the substrate on the formation of epoxides, there were statistically significant
391 differences both among fatty acid methyl esters ($F_{2.213}$ = 43.3, $p < 0.05$) and fatty acids ($F_{2.213}$ =
392 11.9, $p < 0.05$). With both classes of substrates, the mono- and diunsaturated substrates formed a
393 homogenous group, from which the triunsaturated substrates differed (Tukey HSD, $p > 0.05$). The
394 average proportions for epoxide formation for the methyl esters were 32.5% ± 6.2%, 33.0% ± 5.2%
395 and 23.6% ± 1.7%, and for the fatty acids, 59.9% ± 5.4%, 58.0% ± 6.4% and 41.4% ± 3.6% in order
396 of increasing unsaturation. One possible reason for the lower epoxide values with methyl linolenate
397 and linolenic acid could be that the epoxides might have reacted further or that the substrate was
398 oxidised via some other mechanism. When summing up the proportions of epoxides and residual
399 substrates (Tables 2 and 3), it was obvious that the sums were close to 100% with the mono- and
400 diunsaturated substrates (ranging from 85.0%–98.8% with methyl esters and 97.6%–122.0% with

401 fatty acids), but that they were much lower with triunsaturated substrates (ranging from 66.7%–
402 80.9% with methyl esters and 74.1%–90.8% with fatty acids). Therefore, it may be that oat POX is
403 not selective in terms of the unsaturation level of the substrate. Earlier, an oat POX gene transferred
404 and expressed in *Pichia pastoris* preferred oleic acid over other unsaturated fatty acids as the
405 substrate, whereas the hydroperoxides of polyunsaturated fatty acids were better oxygen donors to
406 the fatty acids than oleic acid hydroperoxide was (Meesapyodsuk et al., 2011), which might
407 influence the reactions with natural oat lipids.
408
409 Observations regarding the selectivity of the products were undertaken for methyl linoleate and
410 linoleic acid. Of the two epoxides built up from methyl linoleate and linoleic acid, there were more
411 12,13-epoxy compounds than 9,10-epoxy compounds, with values of 17.9% ± 2.8% and 15.1% ±
412 2.4% for methyl linoleate, and 29.9% ± 3.5% and 28.1% ± 3.1% for linoleic acid. The differences
413 were not big, although they were statistically significant (t_{71} = 41.1, $p < 0.05$ and t_{71} = 9.7, $p < 0.05$).
414 This indicates that both epoxides are likely to be observed in comparable amounts in oat. Similarly,
415 Piazza et al., (2003) reported the formation of an equal amount of mono 9,10-epoxy and 12,13-
416 epoxy octadecenoic acids, thus indicating that the oat POX cannot distinguish the double bonds in
417 the substrate.
418
419 No epoxide formation could be found in any faba bean samples using methyl oleate, oleic acid or
420 triolein as the substrate. Therefore, it was concluded that there was no POX activity or that the
421 activity was very low in the faba bean samples.
422
423 *3.5. Differences in peroxygenase activity among oat cultivars and sample years*
424 There was a statistically significant difference in POX activities among oat cultivars ($F_{3.420}$ = 6.2, p
425 < 0.05) and the values could be clearly divided to produce two homogenous groups (Tukey HSD, p

426 < 0.05). Cultivars Akseli and Meeri belonged to the low POX activity group, with average values of
427 36.9% ± 11.8% and 40.8% ± 14.5%, respectively. The cultivar Meeri also belonged to the high
428 POX group, together with cultivars Alku and Steiner, with values of 43.8% ± 14.6% and 44.1% ±
429 15.2%, respectively. The variation in these average values was large due to the many substrates, but
430 the differences among the cultivars were clear. Epoxidation was always the lowest in cultivar
431 Akseli with all substrates and also in every sample year except for one. Usually, cultivars Steinar
432 and Meeri gave the highest amounts of epoxides and also the lowest residual substrate values. The
433 average activity in cultivar Steiner was 20% greater than in Akseli. The sample year did not have a
434 statistically significant effect on POX activities in the oat samples ($F_{2.420} = 0.98$, $p > 0.05$) and nor
435 was there an interaction between the cultivar and the sample year ($F_{6.420} = 0.72$, $p > 0.05$). Yet a
436 variation in the POX activities among sample years with individual substrates could be observed.
437
438 *3.6. Evaluation of the potential of lipid-modifying enzymes to cause off-flavours*
439 Based on the enzyme activities of three different lipid-modifying enzymes in oat and faba bean, a
440 scheme on off-flavour formation was created (Fig. 4). This study showed that enzymatic activity to
441 hydrolyse lipids occurs both in oat and in faba bean, which may start the lipid oxidation cascade. In
442 oat, due to very low LOX activities, fatty acids are primarily subjected to autoxidation, yielding the
443 formation of hydroperoxides and their further reactions. Anyhow, oxidation of oat lipids occurred
444 much faster when its lipids were being hydrolysed by lipase (Lampi, Damerau, Moisio, Partanen,
445 Forssell & Piironen, 2015). Hydroperoxides of fatty acids are good substrates for oat POX, which
446 could result in the production of epoxy and hydroxy fatty acids, and possible bitter off-flavours in
447 oat products. Since lipid oxidation is initiated by autoxidation, the formation of epoxy and hydroxy
448 fatty acids is not rapid and is more likely to occur when oat or oat products with enzyme activities
449 are stored for longer periods. The high content of lipids and the degree of unsaturation in oat
450 enhances the risk of the formation of epoxy and hydroxy compounds. The very high lipid-

hydrolysing activity present in faba bean, subjects not only the lipids of the seeds, but also of other lipids in the products, to the formation of FFAs. This should be taken into account when e.g. faba bean emulsions are prepared. As the polyunsaturated fatty acids are substrates for faba bean LOX, fatty acid hydroperoxides are built up rapidly and may decompose into volatile oxidation products causing off-flavours. Thus, the so-called beany flavour may be observed rapidly after faba bean flour is suspended in aqueous systems. It is likely that epoxy and hydroxy fatty acids are not as important in terms of producing off-flavours in faba bean as they are in oat products due to the low POX activity in faba bean.

In both seeds, controlling lipid-modifying enzyme activities is crucial in the production of foods of good and stable quality. Differences in the enzyme activities among oat and faba bean cultivars and sample years were observed, but the differences were only modest and would probably not have major effects on off-flavour formation in oat and faba bean.

Acknowledgements

The authors would like to thank Miikka Olin for his skillful technical assistance on the instrument analysis, Boreal Plant Breeding Ltd. for providing the oat samples and Frederick Stoddard for providing the faba bean samples of the cultivars. We would also thank China Scholarship Council for funding the Ph.D studies of Z. Yang.

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543

Figure Legends

Fig. 1. Lipase and LOX activities in oat and faba bean samples from four cultivars in different cultivation years (2 extracts x 3 measurements; $n = 6$): (a) lipase activity in oat; (b) lipase activity in faba bean; and (c) LOX activity in faba bean.

Fig. 2. Combined GC-FID chromatograms of methyl oleate, methyl linoleate, and methyl linolenate after incubation with oat extracts containing POX and formation of epoxides. Where, 1 = methyl oleate, 2 = methyl linoleate, 3 = methyl nonadecanoic acid (internal standard), 4 = methyl linolenate, 5 = methyl 9,10-epoxyoctadecanoate, 6 = methyl 12,13-epoxy-octadec-9-enoate, 7 = methyl 9,10-epoxy-octadec-12-enoate and 8 = epoxides produced from methyl linolenate.

Fig. 3. Combined UHPLC-ELSD chromatograms of oleic acid, linoleic acid, and linolenic acid after incubation with oat extracts containing POX and formation of epoxides. Where, 1 = epoxides produced from linolenic acid, 2 = 12,13-epoxy-9-octadecenoic acid, 3 = 9,10-epoxy-12-octadecenoic acid, 4 = (*trans*-) 9,10-epoxystearic acid, 5 = linolenic acid, 6 = linoleic acid, 7 = oleic acid and 8 = nonadecanoic acid (internal standard).

Fig. 4. The most probable enzymatic and chemical reactions to cause potential off-flavour compounds from lipids in a) oat and b) faba bean.

563 **Table legends**

564 **Table 1**. Identification of the epoxides formed from a) fatty acid methyl esters and b) fatty acids,
565 during incubation with oat extracts containing POX from using GC-MS analysis for fatty acid
566 methyl esters, and UHPLC-Q-TOF for free fatty acids.

567

568 **Table 2**. Proportions of epoxidation products[§] and substrate residues[#] of the three unsaturated fatty
569 acid methyl esters incubated with oat extracts containing POX from four oat cultivar samples from
570 different years (n = 6).

571 [§] Proportions (%) of epoxides from methyl oleate: methyl 9,10-epoxyoctadecanoate;
572 from methyl linoleate: 1= methyl 12,13-epoxy-octadec-9-enoate and 2: methyl 9,10-
573 epoxy-octadec-12-enoate; and methyl linolenate: a mixture of methyl mono-epoxy-
574 octadecadienoates.

575 [#] Proportions (%) of fatty acid methyl esters left after incubation.

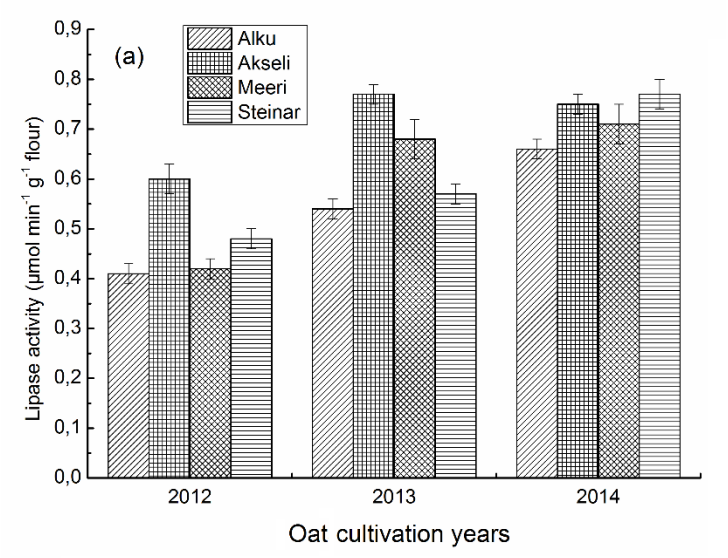
576

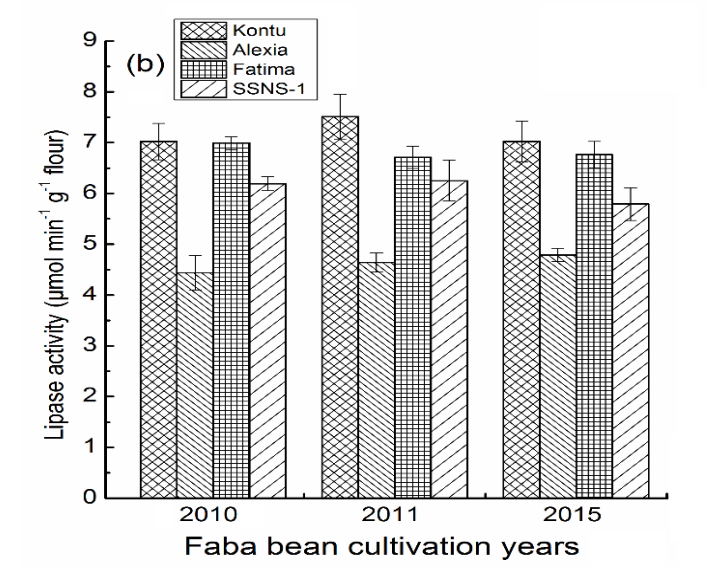
577 **Table 3.** Proportions of epoxidation products[§] and substrate residues[#] of the three unsaturated fatty
578 acids incubated with oat extracts containing POX from four oat cultivar samples from different
579 years (n = 6).

580 [§] Proportions (%) of epoxides from oleic acid: 9,10-epoxyoctadecanoic acid; from
581 linoleic acid: 1= 12,13-epoxy-octadec-9-enoic acid and 2: 9,10-epoxy-octadec-12-
582 enoic acid; and linolenic acid: a mixture of mono-epoxy-octadecadienoic acids.

583 [#] Proportions (%) of fatty acids left after incubation.

Figure 1.


(a)
Alku
Akseli
Meeri
Steinar
Lipase activity (µmol min-1 g-1 flour)
0,0
0,1
0,2
0,3
0,4
0,5
0,6
0,7
0,8
0,9
2012
2013
2014
Oat cultivation years


(b)
Kontu
Alexia
Fatima
SSNS-1
Lipase activity (µmol min-1 g-1 flour)
0
1
2
3
4
5
6
7
8
9
2010
2011
2015
Faba bean cultivation years

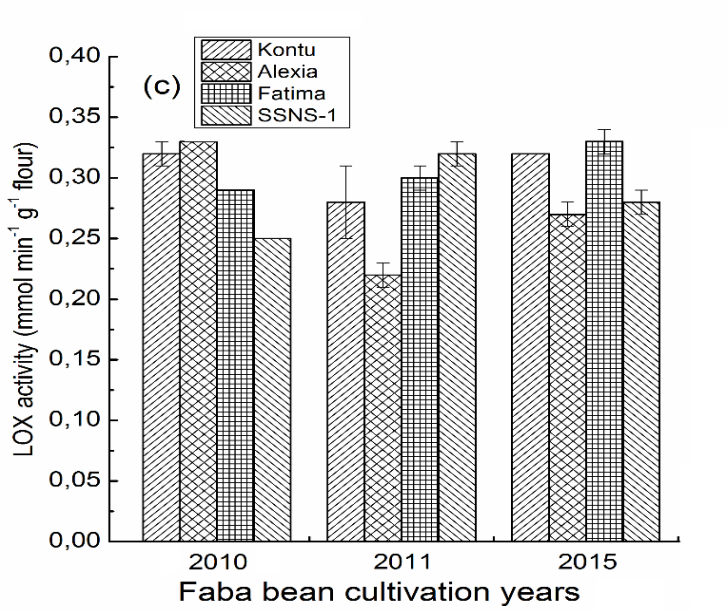

(c)
Kontu
Alexia
Fatima
SSNS-1
LOX activity (mmol min-1 g-1 flour)
0,00
0,05
0,10
0,15
0,20
0,25
0,30
0,35
0,40
2010
2011
2015
Faba bean cultivation years

Figure 2.

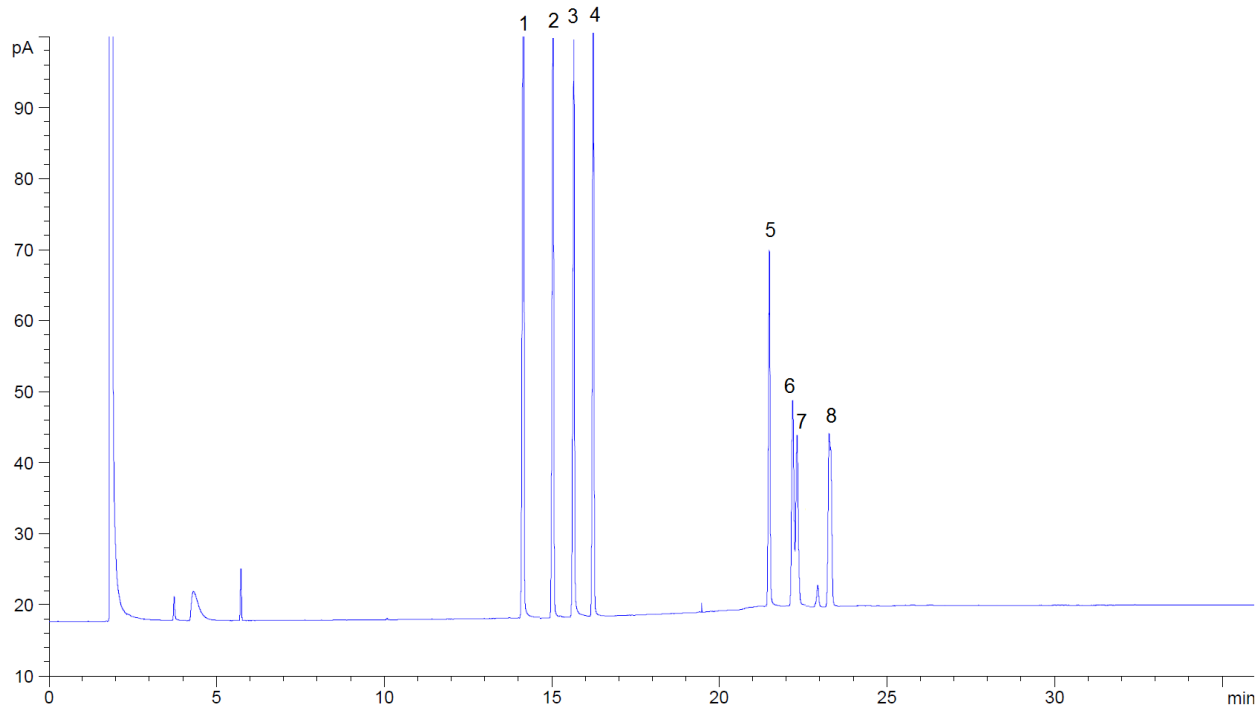


Figure 3.

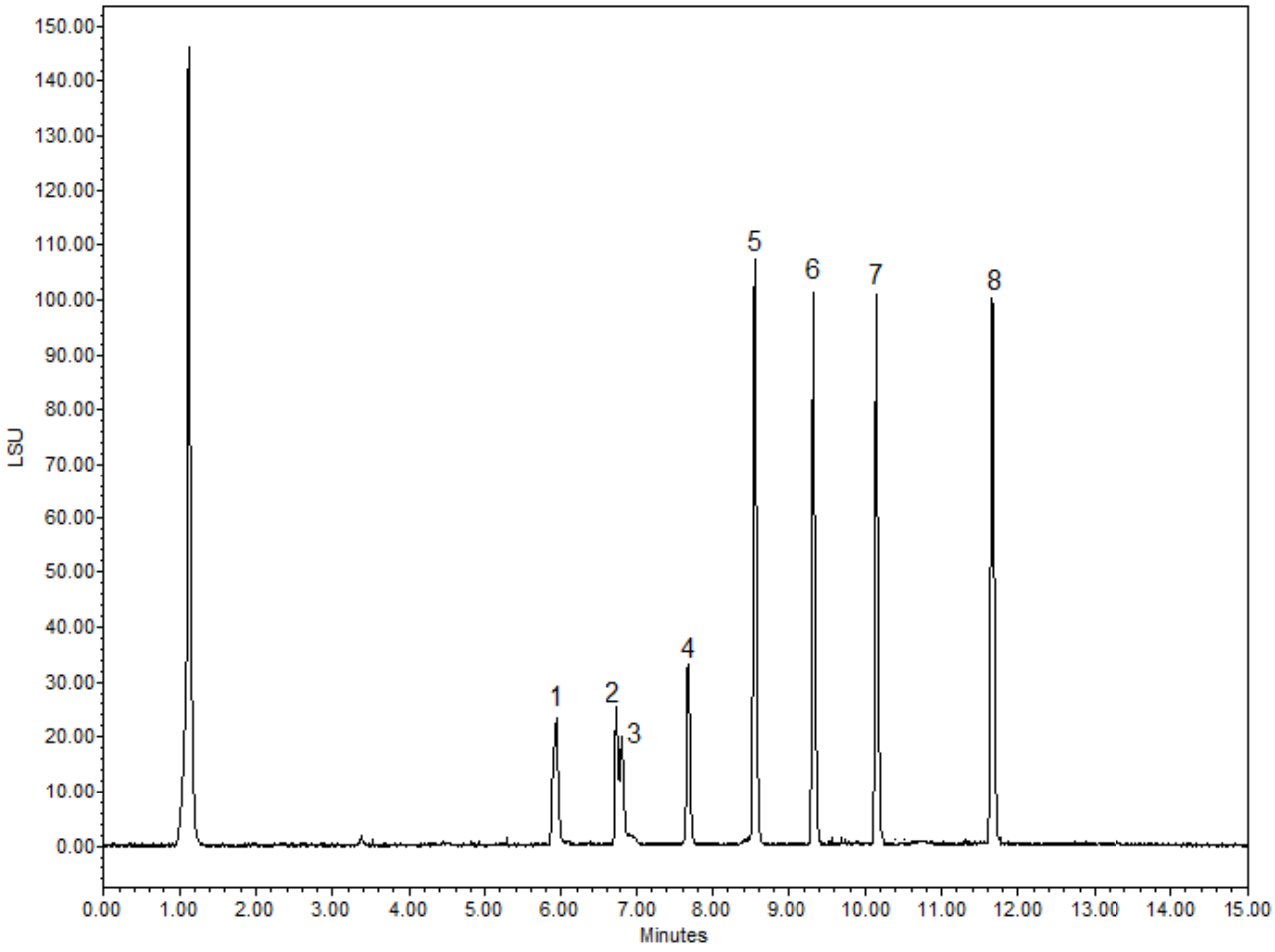


Figure 4.

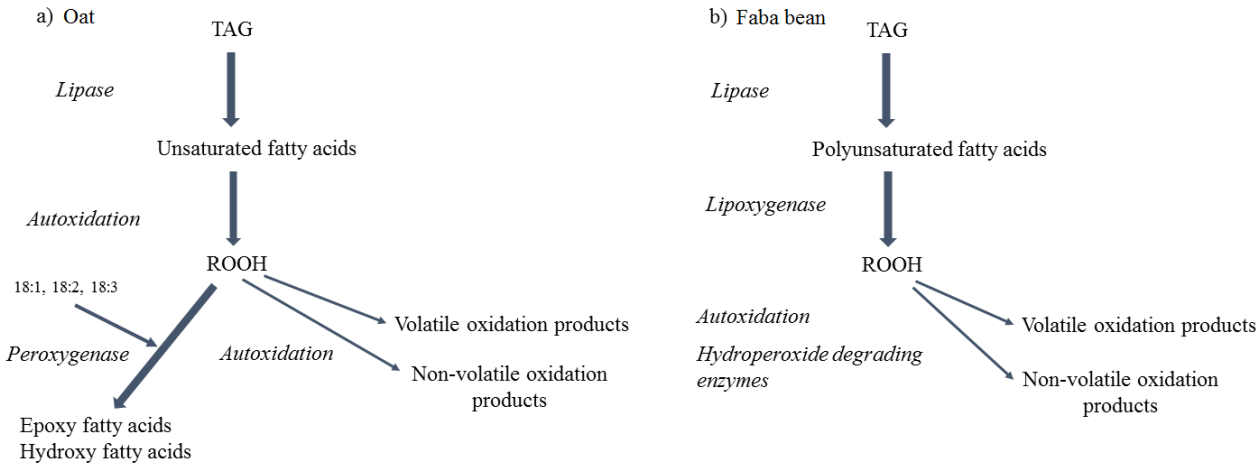

a) Oat
TAG
Lipase
Unsaturated fatty acids
Autoxidation
ROOH
18:1, 18:2, 18:3
Peroxygenase
Autoxidation
Volatile oxidation products
Non-volatile oxidation products
Epoxy fatty acids
Hydroxy fatty acids
b) Faba bean
TAG
Lipase
Polyunsaturated fatty acids
Lipoxygenase
ROOH
Autoxidation
Hydroperoxide degrading enzymes
Volatile oxidation products
Non-volatile oxidation products

Table 1.

Table 1a)

Fatty acid methyl ester epoxide	**R_r : $t_{epoxide}$ / $t_{19:0me}$**	**Molecular ion M^+ (*m/z*)**	**Fragment ions (*m/z*) formed from cleavage of**			**Other fragment ions (*m/z*)**
			the methanol group	**α C-C bond next to the epoxide**		
me-9,10-epoxy-octadecanoate	1.332	312		155; [M-157] $\bullet(CH_2)_7COOCH_3$	199; [M-113] $\bullet(CH_2)_7CH_3$	171
me-12,13-epoxy-octadec-9-enoate	1.354	310	279; [M-31] $\bullet OCH_3$	164; [M-146] C_7OH_{15} and $\bullet OCH_3$	207; [M-103] $CH_3(CH_2)_3CH_3$ and $\bullet OCH_3$	121, 136, 149, 166
me-9,10-epoxy-octadec-12-enoate	1.361	310	279; [M-31] $\bullet OCH_3$		168; [M-142] C_8H_{15} and $\bullet OCH_3$	121, 133, 150, 155, 185
me-epoxy-octadecadienoate	1.423	308	277; [M-31] $\bullet OCH_3$			121, 133, 135, 155, 207, 236

Table 1b)

Fatty acid epoxide	**R_r : $t_{epoxide}$ / $t_{19:0}$**	**Precursor ion $[M\text{-}H]^-$, (*m/z*)**	**Fragment ions (*m/z*) formed from**			
			Loss of H_2O	**Homolytic cleavage of the epoxidic C-O bond followed by scission of the C-C bond**		
				of the epoxide on the side of fatty acid		**on the side of the γ-double bond after hydrogen atom rearrangement**
				$-COO^-$	**$-CH_3$**	
9,10 epoxy-octadecanoic acid	0.649	297.2	279.2	155.1	171.1	
12,13-epoxy-octadec-9-enoic acid	0.575	295.2	277.2	195.1		183.1
9,10-epoxy-octadec-12-enoic acid	0.581	295.2	277.2		171.1	183.1
9,10-epoxy- 12,15-octadeca-dienoic acid	0.532	293.2	275.2		171.1	183.1
15,16-epoxy-9,12-octadeca-dienoic acid	0.537	293.2	275.2	235.2		223.2
12,13-epoxy-9,15-octadeca-dienoic acid	0.549	293.2	275.2	195.1	211.1	223.1; 183.1

Table 2.

Oat Samples	Fatty acid methyl ester substrates						
	me-oleate (%)		me-linoleate (%)			me-linolenate (%)	
Year 2012	**Epoxide[§]**	**Residue[#]**	**Epoxide 1[§]**	**Epoxide 2[§]**	**Residue[#]**	**Epoxides[§]**	**Residue[#]**
Alku	36.2 ± 0.5	59.4 ± 0.3	18.9 ± 0.1	16.3 ± 0.1	50.1 ± 0.3	25.7 ± 0.4	44.8 ± 2.0
Akseli	19.5 ± 0.3	78.2 ± 0.7	11.9 ± 0.3	10.4 ± 0.2	67.0 ± 0.7	24.1 ± 0.2	49.1 ± 0.5
Steinar	38.3 ± 0.4	56.1 ± 0.2	20.1 ± 0.0	17.1 ± 0.1	49.7 ± 0.1	25.5 ± 0.3	46.6 ± 0.5
Meeri	38.2 ± 1.2	57.6 ± 1.7	20.9 ± 0.0	17.7 ± 0.1	48.0 ± 0.4	26.5 ± 0.3	44.3 ± 0.7
Year 2013							
Alku	35.0 ± 1.7	60.6 ± 2.4	19.0 ± 0.3	15.8 ± 0.3	53.0 ± 0.8	20.6 ± 0.3	50.4 ± 1.1
Akseli	27.9 ± 1.1	69.2 ± 0.9	15.2 ± 0.2	12.7 ± 0.2	62.3 ± 0.2	22.2 ± 0.6	51.6 ± 0.5
Steinar	41.6 ± 0.4	54.0 ± 0.3	21.8 ± 0.5	18.7 ± 0.5	46.7 ± 0.4	22.6 ± 0.6	44.7 ± 1.0
Meeri	29.6 ± 0.7	67.3 ± 0.3	17.1 ± 0.2	14.8 ± 0.2	59.1 ± 1.1	23.0 ± 0.5	49.4 ± 1.0
Year 2014							
Alku	30.1 ± 0.4	67.8 ± 0.8	16.0 ± 0.3	13.3 ± 0.2	61.6 ± 0.8	23.5 ± 0.6	54.0 ± 1.4
Akseli	24.2 ± 0.3	74.2 ± 0.7	15.0 ± 0.2	12.4 ± 0.2	63.8 ± 0.7	22.1 ± 0.8	58.0 ± 0.4
Steinar	33.3 ± 0.4	60.9 ± 0.2	19.6 ± 0.2	15.9 ± 0.1	54.2 ± 0.7	23.2 ± 0.7	51.2 ± 0.8
Meeri	35.8 ± 0.4	58.3 ± 0.2	19.7 ± 0.3	16.1 ± 0.2	52.5 ± 0.2	24.4 ± 1.0	51.0 ± 1.3
Total	32.5 ± 6.2	63.6 ± 7.4	17.9 ± 2.8	15.1 ± 2.4	55.7 ± 6.6	23.6 ± 1.7	49.6 ± 4.1

Table 3.

Oat Samples	Free fatty acid substrates						
	Oleic acid (%)		Linoleic acid (%)			Linolenic acid (%)	
Year 2012	**Epoxide[§]**	**Residue[#]**	**Epoxide 1[§]**	**Epoxide 2[§]**	**Residue[#]**	**Epoxides[§]**	**Residue[#]**
Alku	67.5 ± 1.3	44.3 ± 1.1	34.1 ± 1.0	32.0 ± 1.2	37.5 ± 0.7	45.8 ± 1.4	37.4 ± 1.8
Akseli	56.8 ± 2.2	59.3 ± 2.5	26.6 ± 0.2	23.7 ± 0.7	51.7 ± 1.8	38.9 ± 0.8	48.2 ± 1.5
Steinar	63.5 ± 1.2	50.7 ± 0.8	32.9 ± 0.7	30.0 ± 1.0	43.7 ± 0.9	42.9 ± 1.2	43.2 ± 0.6
Meeri	64.0 ± 1.5	50.3 ± 0.6	30.4 ± 0.9	29.0 ± 0.7	42.5 ± 1.0	43.0 ± 0.7	42.5 ± 0.7
Year 2013							
Alku	60.4 ± 1.6	48.3 ± 1.2	31.2 ± 1.2	30.3 ± 0.4	43.5 ± 0.8	43.3 ± 0.6	37.8 ± 0.8
Akseli	51.9 ± 2.3	59.8 ± 1.1	24.4 ± 1.4	23.9 ± 0.5	53.1 ± 0.5	35.8 ± 0.9	47.4 ± 1.5
Steinar	67.4 ± 0.6	42.1 ± 1.4	35.2 ± 0.7	32.3 ± 1.5	37.7 ± 1.0	47.1 ± 1.6	37.5 ± 0.6
Meeri	56.2 ± 1.2	55.8 ± 0.9	26.7 ± 0.2	26.4 ± 0.7	50.6 ± 0.9	39.9 ± 1.8	47.1 ± 1.2
Year 2014							
Alku	58.2 ± 1.3	49.4 ± 3.1	30.5 ± 1.3	28.3 ± 0.9	44.0 ± 1.1	43.2 ± 1.7	43.3 ± 1.5
Akseli	50.7 ± 2.9	59.4 ± 2.2	24.9 ± 1.1	23.8 ± 1.1	53.8 ± 0.9	35.8 ± 1.2	48.6 ± 1.1
Steinar	60.9 ± 1.1	46.8 ± 1.0	30.3 ± 1.0	28.1 ± 0.8	43.0 ± 0.4	39.7 ± 1.1	35.8 ± 0.6
Meeri	61.1 ± 1.1	43.4 ± 1.4	31.8 ± 0.9	29.2 ± 1.0	39.0 ± 1.0	41.0 ± 1.0	36.1 ± 1.1
Total	59.9 ± 5.4	50.8 ± 6.3	29.9 ± 3.5	28.1 ± 3.1	45.0 ± 5.7	41.4 ± 3.6	42.1 ± 4.9